Flavonoids Modulate Bacterial Endophyte Communities of Ginkgo Biloba Leaves

Fangying Lei

Central South University School of Minerals Processing and Bioengineering

Haonan Huang

Central South University School of Minerals Processing and Bioengineering

Qin Yang

Central South University School of Minerals Processing and Bioengineering

Shaodong Fu

Central South University School of Minerals Processing and Bioengineering

Xue Guo

Central South University School of Minerals Processing and Bioengineering

Shuangfei Zhang

Central South University School of Minerals Processing and Bioengineering

Yunhua Xiao

Hunan Agricultural University of Biosciences and Biotechnology

Xueduan Liu

Central South University School of Minerals Processing and Bioengineering

Huaqun Yin

Central South University School of Minerals Processing and Bioengineering

Kai Zou

Central South University School of Minerals Processing and Bioengineering

Yili Liang (✉ liangyili6@csu.edu.cn)

Central South University School of Minerals Processing and Bioengineering <https://orcid.org/0000-0002-8075-5408>

Research Article

Keywords: flavonoids, 16S rRNA, bacterial endophyte communities, Ginkgo biloba, community assembly

Posted Date: June 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-391071/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Flavonoids modulate bacterial endophyte communities of *Ginkgo biloba* leaves

Fangying Lei^{a,b,[+]}, Haonan Huang^{a,b,[+]}, Qin Yang, Shaodong Fu^{a,b}, Xue Guo^{a,b}, Shuangfei Zhang^{a,b}, Yunhua Xiao^c, Xueduan Liu^{a,b}, Huaqun Yin^{a,b}, Kai Zou^{a,b,**}, Yili Liang^{a,b*}

^aSchool of Minerals Processing and Bioengineering, Central South University, Changsha 410083, China

^bKey Laboratory of Biometallurgy, Ministry of Education, Changsha, Hunan, China

^cCollege of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, China

^[+] Authors contribute equally to this work.

*Correspondence:

Yili Liang, School of Minerals Processing and Bioengineering, Central South University, Changsha 410083, China.

E-Mail: liangyili6@csu.edu.cn

Phone: +8615116324959

Fax: +86-0731-88830546

**Correspondence:

Kai Zou, School of Minerals Processing and Bioengineering, Central South University, Changsha 410083, China.

E-Mail: zoukai3412085@hotmail.com

ABSTRACT

Plant-specialized secondary metabolites have ecological functions in mediating interactions between plants and their endophytes. Here, we aimed to reveal the interaction between flavonoid synthesis and endophytic bacterial communities in wild *Ginkgo* trees spanning 100-800 years. We found that flavonoids including quercetin, kaempferol and isorhamnetin decreased while the microbial diversity in leaves increased with the age of sampled trees. Older trees had more unique genera and shifted their endophytic bacterial community structure. Also, Mantel tests and correlation analysis indicated a generally significant ($p < 0.05$) negative correlation between endophytic bacterial communities and flavonoids. Additionally, both deterministic and stochastic processes could play roles in the assembly of endophytic bacterial communities in *Ginkgo* trees with a progressive increase in stochastic processes as flavonoid concentrations decreased. This study provides a mechanistic understanding of how flavonoids modulate the endophytic microbial community assembly.

KEYWORDS: flavonoids; 16S rRNA; bacterial endophyte communities; *Ginkgo biloba*; community assembly

INTRODUCTION

Endophytes, a large number of microbes live inside the host plant without significant infection symptoms in certain or all stages of its life cycle [1]. Those microbes establish a symbiotic relationship with their host plant and play a very important role in the growth and survival of their host through a variety of mechanisms [2]. Consequently, they have applications in agricultural production and environmental restoration [3]. Moreover, because of co-evolving with their host plant, some taxa possess the capacity of producing the same or similar bioactive metabolites of therapeutic importance to the host [4, 5]. Therefore, endophytes received much attention as an integral part of their host's biology in recent years. It is necessary to study plant-associated endophytic communities and their relationships with their host plant.

Host plants present selective pressure on the endophytic community and modulate microbiome assemblage. Usually, host plants obtain their endophytic microbe vertically via the parents, and transmitted microorganisms could move from the spermosphere to the rhizosphere or the phyllosphere, as well as plant tissues, the endosphere [6, 7]. So, a plant of certain genotype located in an environment/ecosystem harbors its own special endophyte composition [8, 9]. The impact of plant genotype on the microbial endophyte community assembly has been reported for crop species and several wild plant species, which confirmed that even intraspecific genetic variation could alter endophyte communities [8, 10]. These studies demonstrated that plant genetic variation affects morphological characteristics such as root growth, architecture, and exudate composition, which in turn impacts the microbial community assembly [9, 11-13]. Furthermore, there were substantial changes in microbial

communities between years or over the course of a growing season, and host age or developmental stages influenced the endophytic microbiome by affecting the expression of plant functional traits including the secretion of blends of compounds and specific phytochemicals [12, 14]. These studies indicated that plant-specialized metabolites have ecological functions, mediating interactions between plants and their endophytic microbiomes.

As a representative of plant functional traits, the synthesis and accumulation of secondary metabolites influence endophytic bacterial community assembly [15]. *Arabidopsis thaliana* produces a range of specialized triterpenes that direct the assembly and maintenance of an *A. thaliana* - specific microbiota within its roots to its own purposes. *In vitro* bioassays with purified triterpene compounds revealed selective growth modulation activities of secondary metabolites toward 19 taxonomically diverse root microbiota. Moreover, some root bacteria were found to be able to selectively metabolize certain triterpenes [16]. Also, the foliar defense phytohormone salicylic acid was required in the biosynthesis and signaling process, to assemble a normal root microbiome in *Arabidopsis*. Salicylic acid modulates colonization of the root by specific bacterial families. The abundance of some root-colonizing bacterial families increased at the expense of others, partly as a function of whether salicylic acid was used as an immune signal or as a carbon source for microbial growth [17]. Similarly, rhizosphere bacteria preferred to consume aromatic organic acids (nicotinic, shikimic, salicylic, cinnamic and indole-3-acetic) exuded by plant roots, which impacted the rhizosphere microbial community assembly in plants (*Avena barbata*) [18]. To date, most studies focused on root microbiota, but the detailed and dynamic mechanism of secondary

metabolites on bacterial endophyte community assembly remains unclear.

Two different processes (deterministic vs. stochastic) are considered to have contributions to species assembly in a local community [19, 20]. Traditional view based on niche theory points that a confluence of deterministic factors, including species traits, interspecific interactions, and environmental conditions, control local community structures [21]. In contrast, neutral theory hypothesizes that all species are ecologically equivalent and the local communities' assembly is actually a random ecological drift. In this process, stochastic processes of birth, death, immigration, emigration and speciation control local community structures, instead of species traits and niches [22]. In recent years, it is more generally accepted that niche theory and neutral theory are not antagonistic in the assembly of a local community, which means both deterministic and stochastic processes simultaneously occur in the community construction [23, 24]. Still, our knowledge about the effect of those two processes on endophytic bacteria assembly is limited.

Ginkgo biloba (*G. biloba*), the most ancient living trees, are widely used as traditional Chinese medicine for centuries, especially for treating cardiovascular and asthma diseases [25, 26], and their secondary metabolites, such as flavonoids and ginkgolides functioning in plant defense and signaling are currently considered as the most prominent pharmacological components [27]. These *Ginkgo*-produced secondary metabolites are found to possess antifungal and antibacterial activities, suggesting potential roles in mediating plant-endophytic microbe interactions [28]. However, associations between secondary metabolites and microbial endophyte communities are deficient in *Ginkgo*.

In this study, we assessed compositional shifts in foliar endophytic bacterial community

of wild *Ginkgo* trees spanning 100-800 years. *Ginkgo* trees grow at a single undisturbed geographical site with little environmental heterogeneity, providing a suitable source for studying the plants and their associated endophytic microbes that have been evolving for hundreds of years. Flavonoids are one of the largest and most structurally diverse families of natural products in *Ginkgo*. The synthesis and accumulation of flavonoids are closely related to the age of *Ginkgo* [29]. We conducted a further investigation to elucidate the mechanism of bacterial endophyte community shift in response to flavonoid content. Here we show that the bacterial endophyte diversity increased with the age of *Ginkgo* trees. There was a negative correlation between bacterial communities and flavonoids. Stochastic processes gradually control community structures as flavonoid contents decreased with the age of sampled trees. This study presents new opportunities to deeply understand plant–bacteria symbioses in *Ginkgo*.

MATERIALS AND METHODS

Site description and sampling

The *Ginkgo biloba* L. trees grow wildly in the national natural reserve of Hupin Mountain in Hunan Province, China. A total of 5 trees were chosen to collect mature leaf samples (Table S1) on 25 Jul. 2016. All trees grew well in mixed forest zones of deciduous and evergreen species, and had a remarkably wide age range (about 100-800 years according to records) and a narrow altitude range (1120-1140 m). The diameter at breast height (DBH) positively correlates with the age of the trees in the same (or similar) environment [30]. So, the girth of the tree trunk was used as a measure of the relative extent of tree ages.

Totally, 40 leaf samples were collected from 5 trees with two sites per tree (the upper and lower part of the crown) and four parallel samples collected from branches of four directions in each site. Each sample was divided into two parts. One was frozen in liquid nitrogen immediately and stored at -80°C for flavonoids extraction after short-time surface cleaning by 75% ethanol and sterile water. To eliminate the presence of surface microorganisms and assess bacterial endophyte diversity and community composition, the other sample was rinsed in flowing water, immersed in 95% ethanol (5 s), rinsed in sterile deionized (5 times), then immersed in 0.5% NaClO (2 min), 70% ethanol (2 min), finally rinsed in sterile deionized H_2O for 5 times. After surface sterilization, all leaves were triturated in liquid nitrogen and stored at -80°C until DNA extraction.

Extraction and determination of flavonoids

Liquid nitrogen-grinded powder (40 meshes) of each leaf sample was extracted in Soxhlet extractor based on Chinese pharmacopeia (2015). 1.000 g powder was homogenized in 100 mL chloroform followed by 2 h reflux at 80°C . The residue was resolved in 50 mL methanol followed by 2 h reflux at 90°C . Then, 20 mL hydrochloride was added to the supernatant, refluxing 30 min at 90°C , and evaporating to dryness. The residue was dissolved in methanol and filtrated through a $0.22\text{ }\mu\text{m}$ membrane for high-performance liquid chromatography (HPLC).

Chromatographic separation was completed on a Shimadzu LC-20AD Series HPLC system (Shimadzu, Duisburg, Germany) equipped with SIL-20A autosampler, SPD-20A UV-VIS detector. ACQUITY UPLCTM BEH C18 column ($217\text{ mm} \times 2.1\text{ mm}$, $1.7\text{ }\mu\text{m}$,

Waters, Milford, USA) was attached to the whole analyses at 25°C. Flavonoids were analyzed with 0.05M sodium acetate (A) and 90% acetonitrile (B) comprising the mobile phase. The gradient elution condition was optimized as follows: (0-20 min, B: 5%-70%; 20-23 min, B: 70%-5%; 23-25 min, B: 5%). A flow rate of 1.0 mL/min was used. Standard curves were established by a series concentration of corresponding standard (ChemFaces, Wuhan, China) at 360 nm. And the content of total flavonoids was calculated by formula [31]:

$$\text{T-flavonoids} = 2.51\text{quercetin} + 2.64\text{kaempferol} + 2.39\text{isorhamnetin}$$

Extraction of endophytic microbial community DNA and sequencing of 16S rRNA gene amplicons

Total DNA was extracted from 0.2 g surface-sterilized leaves grinded with liquid nitrogen using DNeasy® Plant Mini Kit (250) (Qiagen, Inc. Duesseldorf, Germany), strictly following the instruction. Both 1% (w/v) TAE-agarose gel stained with EB and NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) were used to detect the quality and concentration of extracted DNA.

The V4 region of the 16S rRNA gene was amplified on a 2720 thermal cycle (Applies Biosystems, Waltham, Massachusetts, USA) using 799F (5'-AAC MGG ATT AGA TAC CCK G-3') and 1115R (5'-AGG GTT GCG CTC GTT G-3') to reduce amplification of chloroplast DNA [32]. A sample-specific barcode sequence was added to the reverse primer to allow pooling multiple samples in one run of Illumina MiSeq sequencing. PCR amplification was performed as follows: an initial denaturation for 1min at 94°C; and 30 cycles of 20 s at 94°C, 25 s at 57°C and 45 s at 68°C, with a final extension of 10min at 68 °C. All amplicons were

assessed by 1.5% (w/v) TAE-agarose gel stained with EB, cleaned using the Gel Extraction Kit D2500 (OMEGA bio-tek, Norcross, Georgia, USA). Each of 200 ng PCR products was combined as a library and sequenced on the Illumina HiSeq 2500 sequencing platform.

Sequencing data processing

Raw 16S rRNA gene sequencing data were processed using the Galaxy pipeline established by Zhou's lab (<http://zhoulab5.rccc.ou.edu/>) at Oklahoma University (OU), USA. To ensure the quality of the sequences, both forward and reverse reads were trimmed based on sequence quality scores using Btrim [33]. After trimming, the forward and reverse reads with 50 to 250bp overlapping were combined to obtain longer sequences. Also, unqualified sequences were removed if they were too short or contained undetermined base 'N'. Following this, potential chimeric sequences were detected and removed by UCHIME. Sequences were then clustered into operational taxonomic units (OTUs) at 97% sequence similarity using UCLUST [34]. Finally, the RDP Classifier was used to assign 16S rRNA sequences to the bacterial taxa (<http://rdp.cme.msu.edu/classifier/classifier.jsp>) with a minimal 50% confidence estimate. Also, it was necessary to filter OTUs classified as mitochondrion or chloroplast in data outputted from Galaxy pipeline and those unclassified OTUs [35, 36]. All sequences produced from Illumina sequencing had been uploaded to sequence read archive (SRA) of NCBI database with numbers from SRR6763377 to SRR6763386.

Sequencing data analysis

Alpha-diversity indexes, including Shannon index (H), Simpson index (D), Simpson evenness (Si) and Pielou evenness (J) were calculated on Institute for Environmental

Genomics (IEG) (<http://ieg.ou.edu/>). Dissimilarity test for identifying the difference of bacterial communities, and Mantel test, as well as Correlation test for inspecting correlation between bacterial community and flavonoids, were also carried out on this website platform.

Non-metric Multidimensional Scaling (NMDS) for observing the changes of bacterial community composition and Stochasticity test based on a null model were implemented in R statistical analysis platform, with ‘Vegan’, ‘ieggr’ and ‘mCAM’ package. A stochasticity ratio close to zero suggests that the assembly of a local community is highly deterministic and niche-based processes play a significant role in controlling community structure. Whereas, the larger stochasticity ratio suggests neutral processes could have strong effects on community assembly [37].

Other statistical analyses were performed by IBM SPSS Statistics 21 software. One-way ANOVA analyses, followed by Duncan test, were used to test the difference in parameters. Generally, a p-value of less than 0.05 was considered to be significant.

RESULTS

Flavonoid content of *G. biloba* leaves

HPLC analysis showed that quercetin, kaempferol and isorhamnetin were the primary flavonoid metabolites in *G. biloba* leaves. The total content of flavonoids ranged from 1160.94 to 5171.04 mg/kg leaves in the samples tested (Fig. 1A) with kaempferol (191.47-907.39 mg/kg leaves) as the highest (Fig. 1C), followed by quercetin (202.27-749.32 mg/kg leaves) (Fig. 1B), and isorhamnetin (68.69-403.46 mg/kg leaves) as the lowest (Fig. 1D). Besides, the contents of flavonoid decreased with the age of the sampled tree and the

upper part of the crown was generally higher than the lower for each tree, reflecting the strong metabolic activity of flavonoid in younger *Ginkgo* and the difference existed in the same tissue. Further analysis showed that the content was significantly different ($P < 0.05$) among 5 *Ginkgo* trees, except for HP3 and HP4, which had the similar kaempferol and isorhamnetin content (the line chart in Fig. 1). Those differences ($P < 0.05$) also appeared in two sites within individual *Ginkgo* trees (the bar chart in Fig. 1). However, the differences between the two sites were obviously less than the differences between trees.

Endophytic bacterial diversity in *G. biloba* leaves

A total of 122,386 high-quality 16S rRNA gene sequences were obtained and randomly subsampled at a depth of 1,048 sequences per sample. Those sequences were clustered into 1,168 bacterial OTUs by UCLUST at the 97% cutoff, and the rarefaction curve indicated such a sequencing depth was enough to cover major taxa of those microbial communities. HP5 had the highest OTU number among those five trees (Fig. 2), and similar results were observed by Chao index (Fig. 2B). Also, alpha-diversity indexes, including Shannon index, Pielou evenness, Simpson index and Simpson evenness were different ($P < 0.05$) among the trees sampled and increased with the tree age, except for HP4, which was around 380 years but had the lowest Shannon index and Pielou evenness compared with the others. Additionally, the average β -diversity increased with the tree age, except HP4 (Fig. 2G). Bray-Curtis dissimilarity test showed that the structure of bacterial communities among trees was apparently different ($P < 0.05$) (Table S2). The differences between the trees were considerably greater than that between two sites (Table S3). In addition, with the increase of

plant age, the bacterial community disparities have widened as indicated by Nonmetric Multidimensional Scaling (NMDS) (Fig. 3). The results indicated that the alpha- and beta-diversity of endophytic microbial communities was relatively high and generally increased with tree age.

Endophytic bacterial community composition in *G. biloba* leaves

The endophytic bacterial community in *G. biloba* leaves was mainly composed of *Proteobacteria* (51.7 - 99.5%), *Firmicutes* (1.0 - 22.5%), *Actinobacteria* (0.16 - 19.04%), and *Bacteroidetes* (0.14 - 6.00%) (Table S4). However, most of those phyla had different abundances in different samples, and the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Alphaproteobacteria* and *Betaproteobacteria* were significantly ($p < 0.05$) more abundant in HP1 and HP2, while *Gammaproteobacteria* showed a significantly ($p < 0.05$) higher relative abundance in HP3 and HP4. Except for a lower *Gammaproteobacteria* abundance, other phyla in HP5 were obviously higher than those in other trees (Table S5). Also, 298 genera were identified from all sequences and the most abundant thirty genera were identified, and most of those genera in HP5 had significantly ($p < 0.05$) different abundances from other trees (Fig. 4). For example, the genus *Pluralibacter* (formerly named as *Enterobacter*) was less than 0.1% in the bacterial community of HP5; however, this genus was dominant in the other four trees. Furthermore, more unique genera were found in HP5, such as *Bacillus*, *Moraxella* and *Xenophilus*, which were hardly found in four other trees. These results indicated that changes of endophytic bacterial community composition were accompanied by the growth and age of their host plants.

Linkages between endophytic bacterial abundances and flavonoid concentrations

To further quantify the environmental influence on the endophytic bacterial community, the relationship between the relative abundance of endophytic bacterial populations and flavonoids was analyzed by the Mantel test. Although no significant effect on the whole community abundance was observed, many phyla, including *Actinobacteria*, *Armatimonadetes*, *Bacteroidetes*, *Chlamydiae*, *Firmicutes*, *Nitrospirae*, *Proteobacteria* and *Verrucomicrobia*, were significantly ($P < 0.05$) correlated with three flavonol glycosides (Table 1). All of them were correlated with isorhamnetin, whereas, independent of quercetin. Additionally, *Actinobacteria* and *Proteobacteria* were also correlated with kaempferol. Further correlation tests showed that most correlations with flavonoids were remarkably negative, except for *Proteobacteria* ($P \leq 0.05$; Table 2), which had a significant ($P \leq 0.05$) positive correlation, and specifically *Brevibacterium*, *Pluralibacter* and *Shimwellia* had a positive correlation with all three flavonol glycosides (Table 3 and 4).

Deterministic and stochastic processes in the endophytic bacterial community assembly

To explore mechanisms of endophytic bacterial community assembly, a null model analysis was conducted to reveal the importance of deterministic and stochastic processes. Both deterministic and stochastic processes played roles in the assembly and succession of foliar endophytic bacterial communities in *G. biloba* leaves (Fig 5.). However, the relative importance of the two processes was different among those five communities of *G. biloba* leaves. For those endophytic bacterial communities from HP1, HP2, HP3 and HP4, the deterministic process (55.9-65%) appeared to play a more important role in controlling

community composition. However, the stochastic process (64.5%) played a more important role in HP5. The results indicated that both deterministic and stochastic processes regulated the bacterial community structure of *G. biloba* leaves, which might be time-dependent.

DISCUSSION

The synthesis and accumulation of secondary metabolites are closely related to the age of plants according to previous reports [30, 38]. We observed significant differences in quercetin, kaempferol and isorhamnetin among five *G. biloba* trees. Also, the content of three flavonol glycosides decreased with the tree age, confirming previous reports that secondary metabolic activity in younger trees was much higher than old trees [39]. Plant activity at the cellular level can be classified as growth (cell division and enlargement) and differentiation (chemical and morphological changes leading to cell maturation and specialization). The growth-differentiation balance (GDB) hypothesis of plant defense is premised upon a physiological trade-off between growth and differentiation processes (e.g., secondary metabolism) [40, 41]. Since resource allocation for plant defense may compete with resource allocation for plant growth [42], the developmental control of flavonoid metabolism related to age may be regarded as a general strategy to ensure maximum plant fitness.

Plant age caused substantial changes in endophytic bacterial diversity (Fig. 1) and community structure (Fig. 2-3) in *G. biloba*, which were correlated with flavonoid content. In line with the previous report, *G. biloba* leaves harbored diverse bacterial communities containing representatives of 298 genera. Also, older leaf communities were composed of several distinct groups, and the endophytic bacterial diversity increased with the *G. biloba*

tree age, and the effect of time on endophytic bacterial community assembly and succession was much stronger than spatial locations (Fig. 4) although spatial locations are well-known to make a great contribution to bacterial community composition [43, 44]. A recent study showed that microbiome composition shifted as plants aged, which shaped the leaf and root microbiomes [12]. Microbial communities, like plant and animal communities, are dynamic and exhibit temporal patterns that can reflect underlying biotic and abiotic processes [45, 46]. It was suggested that time itself usually is not a driver of community assembly processes [47, 48]. We suggest that physiochemical characteristics of the host *Ginkgo* are important in determining the structure and diversity of the endophytic bacterial community.

Secondary metabolites, a distinct type of natural compounds, can direct the assembly of specific bacterial communities [13, 49]. In this study, Mantel tests and correlation analysis at the phylum and genus levels showed significantly negative correlations between endophytic bacterial communities and flavonol glycosides in the leaves, especially isorhamnetin, perhaps because isorhamnetin possesses high toxicity [50]. These results suggested a role for flavonoids in the *G. biloba* endophytic microbiome assembly. A piece of evidence showed that bacterial communities change in response to salicylic acid signaling in the root zone of *Arabidopsis* [17]. Comparison with the root bacterial profiles of taxonomically remote species supports a role for a triterpene biosynthetic network in mediating the establishment of *Arabidopsis*-specific microbiota [16]. Host plant metabolites can also selectively regulate the growth of bacteria from different taxa by acting as antibiotics or proliferating agents, and plant exudation traits and microbial substrate uptake traits could interact to yield specific patterns of microbial community assembly [18]. For example, scopoletin selectively inhibits

soil-borne fungal pathogens, while growth-promoting rhizobacteria are highly tolerant to scopoletin [13]. Flavonoids may selectively inhibit the growth of bacteria by acting as antibiotics [28]. However, it is possible that some bacteria are highly tolerant to antibiotics and may join forces to trigger flavonoid metabolism [4, 5] as we observed that some genera including *Brevibacterium*, *Pluralibacter* and *Shimwellia*, had positive correlations with all three flavonol glycosides in this study (Table 3 and 4).

A series of *G. biloba* trees growing at a single geographical site for hundreds of years provided an opportunity to test the relative roles of deterministic vs. stochastic processes in long-time disturbed endophytic bacterial community assembly. In the early stage, the endophytic bacterial community was mainly controlled by deterministic processes by strong environmental selection (e.g., high flavonoid concentrations in *G. biloba* leaves), while stochastic process played a more important role in the endophytic bacterial community as the tress aged (especially 800 years old). Based on stochastic factors, the extinction risk of individuals would occur in natural populations [51, 52]. The importance of stochastic processes in the microbial community assembly, at least to some degree, was reflected in the decrease of relative abundances of certain phyla (*Proteobacteria*) and genera (*Pluralibacter*) observed in the HP5 endophytic bacterial community (Fig. 2). Also, an increase in β -diversity in older trees was visualized as the support for important stochastic processes in the microbial community assembly [53, 54]. Because all host plants live in the same mountain with a narrow altitude range (1120-1140 m, a neglected geographical distance), dispersal was not a major restrictive factor affecting the assembly of an endophytic local community. Thus, the endophytic bacterial community changes observed in this study were most likely controlled

by selection (deterministic processes), drift (stochastic processes) or/and speciation (stochastic and deterministic processes). Additionally, a recent work about the succession of soil bacterial communities in a salt marsh over 100 years reported that deterministic selection was positively correlated with sodium concentration [55]. We tended to assume the increase in stochastic processes with the age of host plants was imposed by gradually weakened selection effects by flavonoids in *G. biloba* leaves. A larger sampling size of *G. biloba* trees and a more comprehensive physiochemical analysis of host plants and their associated microbiomes may better understand endophytic microbial community assembly mechanisms in the further.

So, this study provides the first insight into the effect of flavonoids and plant age on bacterial endophytes in wild *G. biloba* leaves. Our results indicated that flavonoids generally decreased, while their microbial diversity increased with the age of sampled trees. Older trees possessed a higher species richness and distinct endophytic bacterial community structure. There was a progressive increase in the stochastic selection of bacterial communities correlated with the decrease of flavonoid concentration. *G. biloba* leaves produce a series of flavonoids that direct the assembly and maintenance of a *Ginkgo* microbiota. More research is needed to reveal detailed mechanisms governing plant microbiome assembly, the possible beneficial functions of the microbial community, and the signaling crosstalk between *Ginkgo* and microbial communities.

ACKNOWLEDGEMENTS

This work was supported by the following grants: Chinese National Science and Technology Support Program (2013BAC09B00), Chinese National Natural Science

Foundation (31570113), and Graduate Research and Innovation Project (1053320170629) in Central South University, China.

AUTHOR CONTRIBUTIONS

FY Lei, HN Huang, YL Liang, K Zou conceived and designed the work. FY Lei, HN Huang, Q Yang, DS Fu performed the experiments. FY Lei and HN Huang wrote and revised the paper. X Guo and SF Zhang helped the sample collection. FY Lei and HN Huang contributed equally to this study. All authors read and approved the final manuscript.

DECLARATION OF INTEREST

All authors declare no competing interests.

REFERENCES

- Schulz BJE, Boyle CJC, Sieber TN. 2006. Microbial root endophytes, pp. 1-13,15-31,33-69,71-87,89-177,179-279,281-319,321-354. In Schulz BJE, Boyle CJC, Sieber TN (eds.), *Microbial Root Endophytes*, Ed.,
- Hardoim PR, van Overbeek LS, van Elsas JD. 2008. Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology*. **16**: 463-471.
- Merzaeva OV, Shirokikh IG. 2010. The production of auxins by the endophytic bacteria of winter rye. *Applied Biochemistry and Microbiology*. **46**: 44-50.
- Cui Y, Yi D, Bai X, Sun B, Zhao Y, Zhang Y. 2012. Ginkgolide B produced endophytic fungus (*Fusarium oxysporum*) isolated from *Ginkgo biloba*. *Fitoterapia*. **83**: 913-920.
- Cao L-L, Zhang Y-Y, Liu Y-J, Yang T-T, Zhang J-L, Zhang Z-G, et al. 2016. Anti-phytopathogenic activity of sporothriolide, a metabolite from endophyte *Nodulisporium* sp A21 in *Ginkgo biloba*. *Pesticide Biochemistry and Physiology*. **129**: 7-13.
- Truyens S, Weyens N, Cuypers A, Vangronsveld J. 2015. Bacterial seed endophytes: genera, vertical transmission and interaction with plants. *Environmental Microbiology Reports*. **7**: 40-50.
- Berg G, Raaijmakers JM. 2018. Saving seed microbiomes. *Isme Journal*. **12**: 1167-1170.
- Bulgarelli D, Garrido-Oter R, Muench PC, Weiman A, Droege J, Pan Y, et al. 2015. Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley. *Cell Host & Microbe*. **17**: 392-403.
- Perez-Jaramillo JE, Carrion VJ, Bosse M, Ferrao LFV, de Hollander M, Garcia AAF, et al. 2017. Linking

- rhizosphere microbiome composition of wild and domesticated *Phaseolus vulgaris* to genotypic and root phenotypic traits. *Isme Journal*. **11**: 2244-2257.
10. Horton MW, Bodenhausen N, Beilsmith K, Meng D, Muegge BD, Subramanian S, *et al.* 2014. Genome-wide association study of *Arabidopsis thaliana* leaf microbial community. *Nat. Commun.* **5**.
  11. Bressan M, Roncato M-A, Bellvert F, Comte G, Haichar FeZ, Achouak W, *et al.* 2009. Exogenous glucosinolate produced by *Arabidopsis thaliana* has an impact on microbes in the rhizosphere and plant roots. *Isme Journal*. **3**: 1243-1257.
  12. Wagner MR, Lundberg DS, del Rio TG, Tringe SG, Dangl JL, Mitchell-Olds T. 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nat. Commun.* **7**.
  13. Stringlis IA, Yu K, Feussner K, de Jonge R, van Bentum S, Van Verk MC, *et al.* 2018. MYB72-dependent coumarin exudation shapes root microbiome assembly to promote plant health. *Proc. Natl. Acad. Sci. U. S. A.* **115**: E5213-E5222.
  14. Chaparro JM, Badri DV, Vivanco JM. 2014. Rhizosphere microbiome assemblage is affected by plant development. *Isme Journal*. **8**: 790-803.
  15. Hu L, Robert CAM, Cadot S, Zhang X, Ye M, Li B, *et al.* 2018. Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nat. Commun.* **9**.
  16. Huang AC, Jiang T, Liu Y-X, Bai Y-C, Reed J, Qu B, *et al.* 2019. A specialized metabolic network selectively modulates *Arabidopsis* root microbiota. *Science*. **364**: 546-+.
  17. Lebeis SL, Paredes SH, Lundberg DS, Breakfield N, Gehring J, McDonald M, *et al.* 2015. Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science*. **349**: 860-864.
  18. Zhalnina K, Louie KB, Hao Z, Mansoori N, da Rocha UN, Shi S, *et al.* 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nat. Microbiol.* **3**: 470-480.
  19. Abrams PA. 2001. The unified neutral theory of biodiversity and biogeography. *Nature*. **412**: 858-859.
  20. Tilman D. 2004. Niche tradeoffs, neutrality, and community structure: A stochastic theory of resource competition, invasion, and community assembly. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 10854-10861.
  21. Fargione J, Brown CS, Tilman D. 2003. Community assembly and invasion: An experimental test of neutral versus niche processes. *Proc. Natl. Acad. Sci. U. S. A.* **100**: 8916-8920.
  22. Chave J. 2004. Neutral theory and community ecology. *Ecol. Lett.* **7**: 241-253.
  23. Gravel D, Canham CD, Beaudet M, Messier C. 2006. Reconciling niche and neutrality: the continuum hypothesis. *Ecol. Lett.* **9**: 399-409.
  24. Ning D, Deng Y, Tiedje JM, Zhou J. 2019. A general framework for quantitatively assessing ecological stochasticity. *Proc. Natl. Acad. Sci. U. S. A.* **116**: 16892-16898.
  25. Zhou W, Chai H, Lin PH, Lumsden AB, Yao QZ, Chen CY. 2004. Clinical use and molecular mechanisms of action of extract of *Ginkgo biloba* leaves in cardiovascular diseases. *Cardiovascular Drug Reviews*. **22**: 309-319.
  26. Birks J, Evans JG. 2009. *Ginkgo biloba* for cognitive impairment and dementia. *Cochrane Database Syst Rev*.
  27. van Beek TA. 2002. Chemical analysis of *Ginkgo biloba* leaves and extracts. *Journal of Chromatography A*. **967**: 21-55.
  28. Cushnie TPT, Lamb AJ. 2005. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*. **26**: 343-356.
  29. Liu X-G, Wu S-Q, Li P, Yang H. 2015. Advancement in the chemical analysis and quality control of flavonoid in *Ginkgo biloba*. *Journal of Pharmaceutical and Biomedical Analysis*. **113**: 212-225.

30. Zou K, Liu X, Zhang D, Yang Q, Fu S, Meng D, *et al.* 2019. Flavonoid Biosynthesis Is Likely More Susceptible to Elevation and Tree Age Than Other Branch Pathways Involved in Phenylpropanoid Biosynthesis in Ginkgo Leaves. *Front. Plant Sci.* **10**.
31. Hasler A, Sticher O, Meier B. 1992. Identification and determination of the flavonoids from Ginkgo biloba by high-performance liquid chromatography. *Journal of Chromatography A.* **605**: 41-48.
32. Chelius MK, Triplett EW. 2001. The diversity of archaea and bacteria in association with the roots of Zea mays L. *Microbial Ecology.* **41**: 252-263.
33. Kong Y. 2011. Btrim: A fast, lightweight adapter and quality trimming program for next-generation sequencing technologies. *Genomics.* **98**: 152-153.
34. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* **27**: 2194-2200.
35. Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics.* **22**: 1658-1659.
36. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microb.* **73**: 5261-5267.
37. Chase JM, Myers JA. 2011. Disentangling the importance of ecological niches from stochastic processes across scales. *Philos. Trans. R. Soc. B-Biol. Sci.* **366**: 2351-2363.
38. Liu Z, Carpenter SB, Bourgeois WJ, Yu Y, Constantin RJ, Falcon MJ, *et al.* 1998. Variations in the secondary metabolite camptothecin in relation to tissue age and season in Camptotheca acuminata. *Tree physiology.* **18**: 265-270.
39. Yao X, Shang E, Zhou G, Tang Y, Guo S, Su S, *et al.* 2012. Comparative Characterization of Total Flavonol Glycosides and Terpene Lactones at Different Ages, from Different Cultivation Sources and Genders of Ginkgo biloba Leaves. *International Journal of Molecular Sciences.* **13**: 10305-10315.
40. Herms DA, Mattson WJ. 1992. The dilemma of plants: to grow or defend. *The quarterly review of biology.* **67**: 283-335.
41. Krome K, Rosenberg K, Dickler C, Kreuzer K, Ludwig-Mueller J, Ullrich-Eberius C, *et al.* 2010. Soil bacteria and protozoa affect root branching via effects on the auxin and cytokinin balance in plants. *Plant and Soil.* **328**: 191-201.
42. Karasov TL, Chae E, Herman JJ, Bergelson J. 2017. Mechanisms to Mitigate the Trade-Off between Growth and Defense. *Plant Cell.* **29**: 666-680.
43. Meyer KM, Leveau JHJ. 2012. Microbiology of the phyllosphere: a playground for testing ecological concepts. *Oecologia.* **168**: 621-629.
44. Leff JW, Del Tredici P, Friedman WE, Fierer N. 2015. Spatial structuring of bacterial communities within individual Ginkgo biloba trees. *Environmental Microbiology.* **17**: 2352-2361.
45. Blasko R, Bach LH, Yarwood SA, Trumbore SE, Hogberg P, Hogberg MN. 2015. Shifts in soil microbial community structure, nitrogen cycling and the concomitant declining N availability in ageing primary boreal forest ecosystems. *Soil Biology & Biochemistry.* **91**: 200-211.
46. Wu Z, Haack SE, Lin W, Li B, Wu L, Fang C, *et al.* 2015. Soil Microbial Community Structure and Metabolic Activity of Pinus elliottii Plantations across Different Stand Ages in a Subtropical Area. *Plos One.* **10**.
47. Park S-J, Cheng Z, Yang H, Morris EE, Sutherland M, Gardener BBM, *et al.* 2010. Differences in soil chemical properties with distance to roads and age of development in urban areas. *Urban Ecosystems.* **13**: 483-497.
48. Hui N, Jumpponen A, Francini G, Kotze DJ, Liu X, Romantschuk M, *et al.* 2017. Soil microbial

- communities are shaped by vegetation type and park age in cities under cold climate. *Environmental Microbiology*. **19**: 1281-1295.
49. Sasse J, Martinoia E, Northen T. 2018. Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? *Trends in Plant Science*. **23**: 25-41.
50. Omidiji O, Ehimedu J. 1990. Changes in the content of antibacterial isorhamnetin 3-glucoside and quercetin 3'-glucoside following inoculation of onion (*Allium cepa* L. cv. Red Creole) with *Pseudomonas cepacia*. *Physiological and molecular plant pathology*. **37**: 281-292.
51. Kendall BE, Fox GA. 2003. Unstructured individual variation and demographic stochasticity. *Conservation Biology*. **17**: 1170-1172.
52. Saether BE, Engen S, Lande R, Moller AP, Bensch S, Hasselquist D, *et al.* 2004. Time to extinction in relation to mating system and type of density regulation in populations with two sexes. *Journal of Animal Ecology*. **73**: 925-934.
53. Chase JM. 2010. Stochastic Community Assembly Causes Higher Biodiversity in More Productive Environments. *Science*. **328**: 1388-1391.
54. Wang J, Shen J, Wu Y, Tu C, Soininen J, Stegen JC, *et al.* 2013. Phylogenetic beta diversity in bacterial assemblages across ecosystems: deterministic versus stochastic processes. *Isme Journal*. **7**: 1310-1321.
55. Dini-Andreote F, Stegen JC, van Elsas JD, Salles JF. 2015. Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. *Proc. Natl. Acad. Sci. U. S. A.* **112**: E1326-E1332.

FIGURES

Fig. 1. Flavonoid content (mg/kg leaves) analysis in *Ginkgo biloba* L. extracts determined by high-performance liquid chromatography (HPLC) using gradient elution at 360nm. (A) The content of total flavonoids. (B) The content of quercetin. (C) The content of kaempferol. (D) The content of isorhamnetin. The error bar was plotted based on the standard deviation of replicate samples ($n = 4$ or 8), and the P -value indicated the statistical significance between the upper part of the crown and the lower within an individual tree.

Fig. 2. Diversity indexes of endophytic bacterial communities within an individual tree: (A) Total OTUs, (B) Chao value, (C) Shannon index, (D) Pielou evenness, (E) Simpson index, (F) Simpson evenness, and within-group β -diversity calculated as the (G) Bray-Curtis

dissimilarity index. All indexes were means with standard deviations (SDs) calculated from eight replicates. The different lower-case letter following the indexes indicates a significant difference ($P<0.05$) between trees.

Fig. 3. Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis distances showing the changes in the composition and an increase β -diversity of the endophytic bacterial community in trees of different ages.

Fig. 4. Endophytic bacterial relative abundance at the genus level.

Fig. 5. Stochasticity test based on a null model for revealing the contribution of stochastic and deterministic processes in the bacterial community. A stochasticity ratio close to zero suggests that the assembly of a local community is highly deterministic and niche-based processes play a significant role in controlling community structure. Whereas, the larger stochasticity ratio suggests that neutral processes are more important. Different letters indicate significant differences ($P<0.05$) between samples.

526

527 **TABLES**

528 **Table 1.** Mantel test showing the relative abundance of endophytic bacterial phyla association
529 with three flavonol glycosides.

530

Phylum	Quercetin		Kaempferol		Isorhamnetin	
	r	p	r	p	r	p
Acidobacteria	-0.079	0.517	0.038	0.283	0.173	0.079
Actinobacteria	-0.015	0.503	0.279	0.004	0.521	0.001
Armatimonadetes	-0.048	0.534	0.118	0.102	0.319	0.002
Bacteroidetes	-0.057	0.674	0.138	0.088	0.310	0.005
Candidatus Saccharibacteria	-0.072	0.459	0.039	0.378	0.165	0.107
Chlamydiae	-0.100	0.800	0.047	0.294	0.209	0.029
Chloroflexi	-0.050	0.302	0.021	0.250	0.104	0.305
Deinococcus-Thermus	-0.080	0.614	-0.015	0.465	0.016	0.429
Firmicutes	-0.091	0.766	0.146	0.063	0.401	0.002
Gemmatimonadetes	-0.067	0.369	0.039	0.388	0.160	0.136
Ignavibacteriae	-0.065	0.367	0.034	0.305	0.149	0.151
Nitrospirae	-0.092	0.730	0.094	0.153	0.300	0.007
Proteobacteria	-0.050	0.563	0.270	0.007	0.572	0.001
Verrucomicrobia	-0.078	0.512	0.047	0.297	0.192	0.048

*Significant differences ($P<0.05$) are indicated in bold.

Table 2. Correlation test showing the relative abundance of endophytic bacterial phyla association with three flavonol glycosides.

Phylum	Quercetin		Kaempferol		Isorhamnetin	
	r	p	r	p	r	p
Acidobacteria	-0.172	0.290	-0.287	0.073	-0.366	0.020
Actinobacteria	-0.267	0.095	-0.481	0.002	-0.660	0.000
Armatimonadetes	-0.185	0.254	-0.344	0.030	-0.452	0.003
Bacteroidetes	-0.252	0.116	-0.389	0.013	-0.582	0.000
Candidatus Saccharibacteria	-0.155	0.341	-0.263	0.101	-0.339	0.032
Chlamydiae	-0.244	0.130	-0.374	0.018	-0.486	0.002
Chloroflexi	-0.112	0.492	-0.185	0.253	-0.236	0.143
Deinococcus-Thermus	-0.151	0.354	-0.091	0.575	-0.160	0.325
Firmicutes	-0.294	0.065	-0.508	0.001	-0.661	0.000
Gemmatimonadetes	-0.160	0.326	-0.264	0.100	-0.335	0.035
Ignavibacteriae	-0.151	0.351	-0.250	0.119	-0.318	0.046
Nitrospirae	-0.248	0.123	-0.415	0.008	-0.531	0.000
Proteobacteria	0.289	0.070	0.501	0.001	0.675	0.000
Verrucomicrobia	-0.187	0.249	-0.309	0.052	-0.396	0.012

*Significant differences ($P<0.05$) are indicated in bold.

Table 3. Mantel test showing the relative abundance of endophytic bacterial genera association with three flavonol glycosides.

Genus (>1%)	Quercetin		Kaempferol		Isorhamnetin	
	r	p	r	p	r	p
<i>Acidovorax</i>	-0.085	0.732	0.144	0.063	0.388	0.002
<i>Acinetobacter</i>	-0.076	0.726	0.191	0.025	0.470	0.001
<i>Actinoplanes</i>	-0.099	0.705	0.114	0.126	0.346	0.001
<i>Amnibacterium</i>	-0.074	0.561	0.052	0.317	0.192	0.047
<i>Atopostipes</i>	-0.080	0.589	0.052	0.292	0.206	0.029
<i>Bacillus</i>	-0.086	0.748	0.160	0.042	0.422	0.002
<i>Blautia</i>	-0.067	0.428	0.034	0.285	0.154	0.126
<i>Brevibacterium</i>	0.329	0.002	0.440	0.001	0.306	0.002
<i>Brevundimonas</i>	-0.093	0.800	0.098	0.178	0.306	0.003
<i>Buttiauxella</i>	-0.054	0.559	0.095	0.165	0.265	0.003
<i>Cloacibacterium</i>	-0.076	0.589	0.087	0.149	0.273	0.006
<i>Clostridium_sensu_stricto</i>	-0.068	0.419	0.174	0.103	0.148	0.106
<i>Comamonas</i>	-0.082	0.798	0.206	0.026	0.490	0.001
<i>Corynebacterium</i>	-0.063	0.687	0.173	0.039	0.446	0.001
<i>Enhydrobacter</i>	-0.090	0.771	0.144	0.064	0.390	0.001
<i>Gaiella</i>	-0.069	0.374	0.036	0.356	0.157	0.135

<i>Geodermatophilus</i>	-0.089	0.700	0.107	0.125	0.319	0.002
<i>Hydrocarboniphaga</i>	-0.064	0.410	0.033	0.280	0.147	0.120
<i>Hymenobacter</i>	-0.076	0.622	0.059	0.290	0.214	0.032
<i>Janibacter</i>	-0.080	0.699	0.124	0.106	0.342	0.001
<i>Kineococcus</i>	-0.074	0.620	0.059	0.276	0.213	0.023
<i>Kineosporia</i>	-0.071	0.551	0.050	0.237	0.191	0.044
<i>Limnohabitans</i>	-0.066	0.612	0.054	0.207	0.190	0.029
<i>Lysinibacillus</i>	-0.059	0.574	0.023	0.262	0.120	0.227
<i>Mangrovibacter</i>	0.013	0.362	0.054	0.200	0.102	0.089
<i>Massilia</i>	-0.084	0.718	0.050	0.273	0.204	0.031
<i>Methylobacterium</i>	-0.092	0.718	0.110	0.111	0.331	0.004
<i>Micromonospora</i>	-0.071	0.504	0.045	0.304	0.177	0.066
<i>Moraxella</i>	-0.080	0.702	0.107	0.184	0.309	0.002
<i>Mycobacterium</i>	-0.099	0.785	0.148	0.063	0.409	0.001
<i>Nocardioides</i>	-0.113	0.826	0.106	0.126	0.349	0.002
<i>Pantoea</i>	-0.101	0.790	0.071	0.245	0.259	0.010
<i>Pedobacter</i>	-0.072	0.480	0.040	0.394	0.167	0.103
<i>Planomicrobium</i>	-0.067	0.370	0.039	0.320	0.160	0.126
<i>Pluralibacter</i>	-0.043	0.545	0.279	0.004	0.578	0.001
<i>Propionibacterium</i>	-0.081	0.769	0.167	0.049	0.430	0.001
<i>Pseudomonas</i>	-0.086	0.712	0.114	0.105	0.330	0.001
<i>Rhizobium</i>	-0.085	0.716	0.104	0.138	0.308	0.002

<i>Rhodococcus</i>	-0.080	0.656	0.061	0.253	0.219	0.022
<i>Shimwellia</i>	0.187	0.023	0.417	0.001	0.644	0.001
<i>Sphingobacterium</i>	0.046	0.238	0.188	0.021	0.327	0.003
<i>Sphingomonas</i>	-0.068	0.678	0.145	0.052	0.374	0.001
<i>Spirosoma</i>	-0.089	0.629	0.053	0.317	0.214	0.030
<i>Staphylococcus</i>	-0.080	0.662	0.111	0.131	0.321	0.002
<i>Stenotrophomonas</i>	-0.080	0.718	0.134	0.085	0.365	0.001
<i>Streptococcus</i>	-0.060	0.616	0.026	0.267	0.127	0.195
<i>Tuberibacillus</i>	-0.065	0.514	0.041	0.251	0.165	0.058
<i>Unclassified</i>	-0.078	0.702	0.141	0.061	0.367	0.001
<i>Xanthomonas</i>	-0.075	0.669	0.087	0.179	0.269	0.007
<i>Xenophilus</i>	-0.085	0.589	0.089	0.174	0.279	0.006

---

*Significant differences ($P < 0.05$) are indicated in bold.

Table 4. Correlation test showing the relative abundance of endophytic bacterial genera association with three flavonol glycosides.

Genus (>1%)	Quercetin		Kaempferol		Isorhamnetin	
	r	p	r	p	r	p
<i>Acidovorax</i>	-0.292	0.067	-0.473	0.002	-0.610	0.000
<i>Acinetobacter</i>	-0.275	0.087	-0.493	0.001	-0.646	0.000
<i>Actinoplanes</i>	-0.263	0.101	-0.435	0.005	-0.555	0.000
<i>Amnibacterium</i>	-0.181	0.263	-0.301	0.059	-0.383	0.015
<i>Atopostipes</i>	-0.190	0.242	-0.317	0.046	-0.408	0.009
<i>Bacillus</i>	-0.285	0.075	-0.489	0.001	-0.633	0.000
<i>Blautia</i>	-0.154	0.342	-0.254	0.113	-0.325	0.041
<i>Brevibacterium</i>	0.430	0.006	0.547	0.000	0.375	0.017
<i>Brevundimonas</i>	-0.274	0.088	-0.464	0.003	-0.590	0.000
<i>Buttiauxella</i>	-0.205	0.204	-0.353	0.025	-0.458	0.003
<i>Cloacibacterium</i>	-0.210	0.193	-0.369	0.019	-0.478	0.002
<i>Clostridium_sensu_stricto</i>	0.000	1.000	0.000	1.000	0.000	1.000
<i>Comamonas</i>	-0.328	0.039	-0.553	0.000	-0.699	0.000
<i>Corynebacterium</i>	-0.236	0.143	-0.461	0.003	-0.605	0.000
<i>Enhydrobacter</i>	-0.294	0.066	-0.479	0.002	-0.613	0.000
<i>Gaiella</i>	-0.158	0.332	-0.261	0.104	-0.332	0.036
<i>Geodermatophilus</i>	-0.260	0.105	-0.430	0.006	-0.551	0.000

<i>Hydrocarboniphaga</i>	-0.148	0.363	-0.245	0.128	-0.313	0.049
<i>Hymenobacter</i>	-0.178	0.272	-0.303	0.057	-0.386	0.014
<i>Janibacter</i>	-0.257	0.109	-0.433	0.005	-0.555	0.000
<i>Kineococcus</i>	-0.191	0.238	-0.318	0.046	-0.409	0.009
<i>Kineosporia</i>	-0.174	0.284	-0.289	0.071	-0.371	0.018
<i>Limnohabitans</i>	-0.186	0.251	-0.293	0.067	-0.377	0.016
<i>Lysinibacillus</i>	-0.118	0.470	-0.199	0.217	-0.257	0.109
<i>Mangrovibacter</i>	-0.233	0.148	-0.174	0.283	0.042	0.799
<i>Massilia</i>	-0.198	0.221	-0.323	0.042	-0.412	0.008
<i>Methylobacterium</i>	-0.257	0.110	-0.432	0.005	-0.554	0.000
<i>Micromonospora</i>	-0.172	0.289	-0.285	0.075	-0.362	0.022
<i>Moraxella</i>	-0.234	0.146	-0.400	0.011	-0.514	0.001
<i>Mycobacterium</i>	-0.290	0.069	-0.485	0.002	-0.621	0.000
<i>Nocardioides</i>	-0.285	0.075	-0.476	0.002	-0.609	0.000
<i>Pantoea</i>	-0.249	0.122	-0.387	0.014	-0.497	0.001
<i>Pedobacter</i>	-0.173	0.285	-0.279	0.082	-0.356	0.024
<i>Planomicrobium</i>	-0.159	0.329	-0.262	0.102	-0.334	0.035
<i>Pluralibacter</i>	0.344	0.030	0.573	0.000	0.734	0.000
<i>Propionibacterium</i>	-0.265	0.098	-0.474	0.002	-0.618	0.000
<i>Pseudomonas</i>	-0.261	0.104	-0.436	0.005	-0.561	0.000
<i>Rhizobium</i>	-0.254	0.114	-0.422	0.007	-0.542	0.000
<i>Rhodococcus</i>	-0.209	0.195	-0.347	0.028	-0.442	0.004

<i>Shimwellia</i>	0.449	0.004	0.621	0.000	0.764	0.000
<i>Sphingobacterium</i>	0.036	0.827	-0.052	0.751	-0.222	0.168
<i>Sphingomonas</i>	-0.240	0.135	-0.430	0.006	-0.565	0.000
<i>Spirosoma</i>	-0.191	0.237	-0.319	0.045	-0.406	0.009
<i>Staphylococcus</i>	-0.231	0.151	-0.398	0.011	-0.523	0.001
<i>Stenotrophomonas</i>	-0.244	0.130	-0.431	0.005	-0.566	0.000
<i>Streptococcus</i>	-0.124	0.446	-0.211	0.192	-0.271	0.090
<i>Tuberibacillus</i>	-0.162	0.320	-0.266	0.097	-0.340	0.032
<i>Unclassified</i>	-0.395	0.012	-0.529	0.000	-0.629	0.000
<i>Xanthomonas</i>	-0.206	0.203	-0.362	0.022	-0.465	0.003
<i>Xenophilus</i>	-0.223	0.166	-0.380	0.016	-0.488	0.001

*Significant differences ($P < 0.05$) are indicated in bold.

Table S1. Location and altitude of sampling trees.

Trees	Longitude	Latitude	Altitude(m)	Girth(m)	Estimated age
HP1	E 110°46' 27"	N 30°06' 34"	1120	0.69	100
HP2	E 110°46' 27"	N 30°06' 34"	1140	1.80	150
HP3	E 110°48' 50"	N 30°05' 47"	1140	3.00	200
HP4	E 110°36' 16"	N 26°55' 13"	1140	4.12	380
HP5	E 110°38' 28"	N 29°56' 07"	1130	5.30	800

Table S2. Bray-Curtis dissimilarity test of endophytic bacterial community among trees.Significant differences ($P < 0.05$) are indicated in bold.

	HP1		HP2		HP3		HP4	
HP2	0.129	0.001						
HP3	0.127	0.001	0.130	0.001				
HP4	0.116	0.001	0.119	0.003	0.117	0.001		
HP5	0.359	0.001	0.362	0.001	0.360	0.001	0.349	0.001

*Significant differences ($P < 0.05$) are indicated in bold.

Table S3. Bray-Curtis dissimilarity test of endophytic bacterial community between two sides within individual trees.

	HP1	HP2	HP3	HP4	HP5
r	0.115	0.13	0.119	0.098	0.55
p	0.026	0.225	0.035	0.023	0.173

*Significant differences ($P<0.05$) are indicated in bold.

585 **Table S4.** The relative abundance of endophytic bacterial communities at the phylum level.

Phylum(%)	HP1	HP2	HP3	HP4	HP5
Acidobacteria	0.00	0.00	0.00	0.00	0.08
Actinobacteria	2.22	1.49	0.16	0.19	19.04
Armatimonadetes	0.01	0.00	0.00	0.00	0.16
Bacteroidetes	1.09	1.42	0.16	0.14	6.00
Candidatus Saccharibacteria	0.00	0.00	0.00	0.00	0.05
Chlamydiae	0.01	0.02	0.01	0.01	0.19
Chloroflexi	0.00	0.00	0.00	0.00	0.01
Deinococcus-Thermus	0.00	0.01	0.00	0.00	0.01
Firmicutes	1.04	0.51	0.21	0.26	22.52
Gemmatimonadetes	0.00	0.00	0.00	0.00	0.02
Ignavibacteriae	0.00	0.00	0.00	0.00	0.04
Nitrospirae	0.00	0.00	0.00	0.00	0.10
Proteobacteria	95.63	96.54	99.46	99.39	51.74
Alphaproteobacteria	0.24	0.11	0.07	0.02	13.69
Betaproteobacteria	0.45	0.27	0.17	0.12	10.95
Deltaproteobacteria	0.00	0.01	0.00	0.00	0.30
Gammaproteobacteria	94.87	96.10	99.20	99.25	26.69
Verrucomicrobia	0.00	0.00	0.00	0.00	0.05

586 *The relative abundance of phyla appearing in all samples are indicated in bold.

Figures

Figure 1

Flavonoid content (mg/kg leaves) analysis in *Ginkgo biloba* L. extracts determined by high-performance liquid chromatography (HPLC) using gradient elution at 360nm. (A) The content of total flavonoids. (B) The content of quercetin. (C) The content of kaempferol. (D) The content of isorhamnetin. The error bar was plotted based on the standard deviation of replicate samples (n = 4 or 8), and the P-value indicated the statistical significance between the upper part of the crown and the lower within an individual tree.

Figure 2

Diversity indexes of endophytic bacterial communities within an individual tree: (A) Total OTUs, (B) Chao value, (C) Shannon index, (D) Pielou evenness, (E) Simpson index, (F) Simpson evenness, and within-group β -diversity calculated as the (G) Bray-Curtis dissimilarity index. All indexes were means with standard deviations (SDs) calculated from eight replicates. The different lower-case letter following the indexes indicates a significant difference ($P < 0.05$) between trees.

Figure 4

Endophytic bacterial relative abundance at the genus level.

Figure 5

Stochasticity test based on a null model for revealing the contribution of stochastic and deterministic processes in the bacterial community. A stochasticity ratio close to zero suggests that the assembly of a local community is highly deterministic and niche-based processes play a significant role in controlling community structure. Whereas, the larger stochasticity ratio suggests that neutral processes are more important. Different letters indicate significant differences ($P < 0.05$) between samples.